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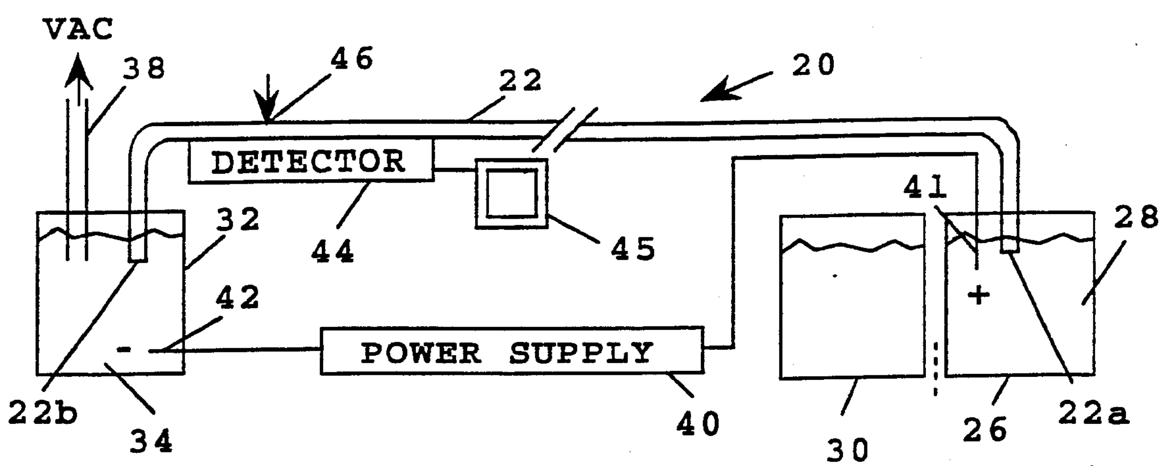

(57) Abstract

A method for achieving desired electroosmotic flow characteristics in a capillary tube (22) having charged surface groups. An electrolyte solution (28) containing a compound effective to stably alter the charge of the tube walls is drawn into and through the tube (22) while the electroosmotic flow rate in the tube (22) is being monitored (44) until a desired electroosmotic flow rate is achieved. The method can be used to optimize electrophoretic separation of charged protein or nucleic acid species in a capillary tube, and to produce capillary tubes with desired charge density properties.

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FLOW-RATE CONTROLLED SURFACE CHARGE COATING FOR CAPILLARY ELECTROPHORESIS

1. Field of the Invention

The present invention relates to capillary electrophoresis, and in particular, to methods for achieving controlled electro-osmotic flow rates in a capillary tube, for enhancing electrophretic separation of biomolecules.

2. References

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3. Background of the Invention

Capillary electrophoresis (CE) has been proposed for rapid fractionation of a variety of biomolecules, including DNA species, proteins, peptides, and derivatized

amino acids (Cohen, 1987, 1988, Compton, Kaspar). Typically, the method employs fused silica capillary tubes whose inner diameters are between about 50-200 microns, and which can range in length between about 10-100 cm or more.

In the usual CE procedure, the capillary tube is filled with an electrophoresis medium, a small sample volume is drawn into one end of the tube, and an electric field is placed across the tube to draw the sample through the medium. The electrophoretic medium may be a non-flowable polymer or gel material, for use in certain types of CE fractionation, or a fluid material which is suitable for other types of CE fractionation. Electrophoretic separation of proteins in a fluid electrophoretic medium, based on the differential charge density of the protein species, has been reported (Lauer). In applying CE techniques to nucleic acid fractionation, where the species to be fractionated high similar charge densities, it has been found that high-resolution fractionation does not require a gelled matrix medium, but may also be achieved in a fluid electrophoretic medium containing high molecular weight polymers (co-owned US patent appication for "Nucleic Acid Fractionation by Counter-Migration Capillary Electrophoresis", Serial No. 390,631, filed August 7, 1989).

When CE is carried out using a fluid electrophoretic medium, the medium itself may undergo bulk flow migration through the capillary tube toward one of the electrodes. This electro-osmotic flow is due to a charge shielding effect produced at the capillary wall interface. In the case of fused silica tubes, which carry negatively charged silane groups, the charge shielding produces a cyclindrial "shell" of positively charged ions in the electrophoresis medium near the surface wall. This

shell, in turn, causes the bulk flow medium to assume the character of a positively charged column of fluid, and migrate toward the cathodic electrode at an electro-osmotic flow rate which is dependent on the thickness (Debye length) of the shell. The rate of electro-osmotic flow of the fluid medium through the tube is also dependent on electric field strength, and viscosity of the medium.

As detailed in the above-cited patent application, electro-osmotic flow rate may provide a important variable which can be optimized to improve separation among two or more similar species. In particular, when CE is carried out under conditions in which electro-osmotic flow and the migration of species to be separated are in opposite directions, the effective column length for separation can be made extemely long, by making the electro-osmotic flow rate in one direction nearly equal to the electrophoretic migration rate of a species in the opposite direction.

Heretofore, attempts to modulate or control electro-osmotic flow rate have been limited. In one approach, the pH of the electrophoretic medium is made sufficiently low, e.g., less than pH 4, to protonate the charged surface groups, and thus reduce surface charge density. This approach is inapplicable to many proteinsm where low-pH denaturation effects can occur.

It has also been proposed to include in the electrophoretic buffer, a charged agent which can bind to the surface at some equilibrium constant to mask suface charge,and thus reduce electro-osmotic flow. This approach is severely limited by the problem of the charged agent binding to the species to be separated, and thus altering the charge density of these species. Also, the concentration of binding compound must be calibrated by

trial and error.

Attempts to reduce or eliminate electro-osmotic flow by covalently derivatizing the charged surface groups with neutral or positively charged agents has also been reported. This approach has not been widely adopted, primarily because of difficulty in calibrating the reaction conditions to achieve a desired electro-osmotic flow, and also because the reaction is irreversible, i.e., the tube cannot be recoated to another selected electro-osmotic flow rate.

4. Summary of the Invention

It is therefore one general object of the invention to provide a method for achieving a selected electro-osmotic flow rate in a CE tube.

A more specific object is to provide such a method which is easily performed, compatible with both protein and nucleic acid CE fractionation, and which may be carried out in a manner which results in either reversible or irreversible surface charge densities in a CE tube.

The invention includes, in one aspect a method of achieving selected electro-osmotic flow characteristics in a capillary tube having charged surface groups. The tube is connected between anodic and cathodic electrolyte reservoirs, and an electric field is placed across the reservoirs to produce electroosomotic flow within the tube. During electro-osmotic flow, a compound capable of altering the surface charge of the tube is drawn into and through the tube, and the electro-osmotic flow rate within the tube is monitored. The compound is continued to be drawn into and through the tube until a desired electro-osmotic flow rate in the tube, as determined from said monitoring, is achieved.

Figure 4 illustrates the principle of flow-rate controlled surface-charge coating (FCSC);

Figure 5 is a plot of electro-osmotic flow rate as a function of coating time with polybrene;

Figure 6 is a plot of electro-osmotic flow rate as a function of coating time using 0.0005% polybrene;

Figure 7 is a plot of electro-osmotic flow rate as a function of coating time using polybrene at two polybrene concentrations;

Figure 8 is a plot of electro-osmotic flow rate as a function of coating time using spermine;

Figure 9 is a plot of electro-osmotic flow rate as a function of coating time with dodecyl trimethyl ammonium bromide;

Figure 10 is an CE electropherogram of lactate dehydrogenase in the absence of polybrene;

Figure 11 is a CE electropherogram of lactate dehydrogenase in a CE tube coated with polybrene;

Figure 12 demonstrates the resolution of five acetylated forms of histone H4 using a polybrene-coated capillary;

Figure 13 illustrates the separation of two species of RNase T1;

Figure 14 shows the electropherogram generated from a capillary electrophoresis run of DNA in the absence of NaCl when the capillary has been coated with polybrene;

Figure 15 shows the electropherogram generated from a capillary electrophoresis run of DNA in the presence of 10 mM NaCl when the capillary has been coated with polybrene;

Figure 16 shows the electropherogram generated from a capillary electrophoresis run of DNA in the presence of 20 mM NaCl when the capillary has been coated with polybrene;

Figure 17 shows the electropherogram generated from a capillary electrophoresis run of two species of RNase T1 in the absence of salt;

Figure 18 shows the resolution of two species of RNase T1 in the presence of 30 mM NaCl.

Detailed Description of the Invention

I. Capillary Electrophoresis System

Figure 1 is a simplified schematic view of a capillary electrophoresis (CE) system 20 suitable for practicing the flow-rate controlled surface-charge coating (FCSC) method of the invention, as well as for carrying out electrophoretic separations using tubes prepared by the FCSC method. The system includes a capillary tube 22 having a length preferably between about 10-200 cm, typically less than about 100 cm, and an inner diameter of preferably between about 25-200 µm (microns), typically about 50 µm. In the embodiment shown, the tube is supported in a horizontal position and has downwardly bent end regions.

The inner surface of the tube has chemical groups which are either negatively or positively charged at the a pH preferably between about 4-9. The surface chemical groups may be an inherent property of the capillary material, such as is the case for a fused silica tube which has surface silane groups resulting in a negative charge. Alternatively, or in addition, the capillary walls may be treated with known derivatization reagents for attachment of chemical groups, such as quaternary amines, to the inner capillary walls, or with known positively charged surface-coating agents. One preferred capillary tube is a fused silica tube having an inner

The electro-osmotic flow rate through the tube is preferably monitored by introducing into the tube, at spaced time intervals, a series of pulses of a flow marker whose travel through the tube can be used to monitor the electro-osmotic flow rate of a band of fluid in the tube containing the marker solution.

In one general embodiment, the tube is a fused silica tube having negatively charged surface silane groups, and the charge-altering compound is a polymer containing regularly spaced, charged amine groups, preferably a hydrophobic polymer with quaternary amine charged groups, such as the polymer polybrene.

In another general embodiment, the capillary tube is a glass tube having positively charged amine groups, and the charge-altering compound is a negatively charged polymer, such as polymer of polysulfonic acid, polycarboxylic acid, polyphosphonic acid, or polyphosphoric acid.

Where a charged, hydrophobic polymer, such as a hydrophobic polyamine, is used, the method may be practiced to achieve a selected degree of overcoating which produces electro-osmotic flow in a reverse direction. In this method, the polymer is first drawn through the tube through in the initial direction of electro-osmotic flow, until the charge on the surface walls is neutralized and electro-osmotic flow in the initial direction ceases. Thereafter, the compound is drawn into and through the tube in the same direction, until electro-osmotic flow within the tube in the opposite direction reaches a selected rate. This method is particularly useful for separating positively charged proteins since (a) the net positive charge of the tube walls is positive and therefore prevents electrostatic protein binding on the wall surfaces, and (b) the reverse-direction rate of electro-

osmotic flow has been selected to optimaize protein separation.

The method may further include a step for producing a capillary tube having a selected density of covalently attached charged groups on the surface of the capillary wall, using a compound which has chemical groups both for masking wall surface charge and for covalent attachment to reactive chemical groups on the tube wall. After achieving the desired electro-osmotic flow, the coated tube is treated with a coupling agent effective to couple the compound covalently to the surface walls.

In another aspect, the invention includes a CE tube formed by the method of the invention. The tube is characterized by a (a) selected electro-osmotic flow, in a given electrophoresis medium, and (b) a coating of a charged polymer agent. In one preferred embodiment, the charged agent is a hydrophobic polyquaternary amine polymer.

These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Figures

Figure 1 is a schematic diagram of a capillary electrophoresis system used in practicing the method of the present invention;

Figure 2 is a schematic view of a capillary electrophoresis system designed for operation simultaneously in both a pulsed and constant-voltage mode;

Figure 3 is an enlarged, fragmentary portion of a capillary electrophoresis tue, illustrating electro-osmotic flow (e) in a right-to-left direction, and fragment migration (m_1, m_2, m_3) in a right-to-left direction;

diameter of 50 µm and available from Polymicro Technologies (Phoenix, AZ).

More generally, the capillary tube may be any tube or channel capable of supporting a column of buffer, preferably at a column thickness of 200 µm or less. For example, the tube may take the form of a channel formed in a glass slide or the like, and having negatively charged surface groups.

A anodic reservoir 26 in the system contains an electrolytic solution 28 which is drawn through the tube by electro-osmotic flow (Section II) with the application of an electric field across the tube ends. The anodic end of the tube, indicated at 22a, is immersed in the solution, as shown, during electrophoresis.

A reservoir 30 in the system may contain a marker solution, for use during the ECSCC method, or may contain a sample of molecules to be separated, during an electrophoretic separation. Preferably the marker or sample material is dissolved in the electrolytic solution or in water. The two anodic reservoirs may be carried on a carousel or the like, for placement at a position in which the lower anodic end of the tube can be immersed in the reservoir fluid. Although not shown here, the carousel may carry additional reservoirs containing solutions for cleaning and flushing the tube between electrophoretic runs or different solutions, where two or more solutions are employed in a single electrophoretic fractionation method.

The opposite, cathodic end of the tube, indicated at 22b, is sealed within a cathodic reservoir 32 and is immersed in an cathodic electrolyte solution 34 contained in the reservoir, as shown. A second tube 38 in the reservoir is connected to a finely-controlled vacuum system (not shown) for drawing fluid, (e.g., washing and

cleaning solutions, marker solution, and electrophoresis buffer solution) through the tube and for loading the macromolecule sample material in reservoir 30 into the tube.

A high voltage supply 40 in the system is connected to the anodic and cathodic reservoirs as shown, for applying a selected electric potential between the two reservoirs. The power supply leads are connected to platinum electrodes 41, 42 in the anodic and cathodic reservoirs, respectively. The power supply may be designed for applying a constant voltage (DC) across the electrodes, preferably at a voltage setting of between 5-50 KV. Alternatively, or in addition, the power supply may be designed to apply a selected-frequency, pulsed voltage between the reservoirs. In general, the shorter the capillary tube, the higher the electric field strength that can be applied, and the more rapid the electrophoretic separation.

When operated in a pulsed voltage mode, the power supply preferably outputs a square wave pulse at an adjustable frequency of about 50 Hz up to a KHz range, and an rms voltage output of about 10-30 KV. Higher pulse frequencies, even into the MHz range may be suitable for some applications.

Completing the description of the system shown in Figure 1, a detector 44 in the system is positioned adjacent the cathodic end of the tube, for optically monitoring nucleic acid fragments migrating through an optical detection zone 46 in the tube. The detector may be designed either for UV absorption detection and/ or for fluorescence emission detection. UV absorbance is typically carried out at 240-280 nm, using, for example, a Kratos 783 UV absorbance detector which has been modified by Applied Biosystems (Foster City, CA.), by repla-

cing the flow cell with a capillary holder. Fluorescence emission detection is preferably carried out at a selected excitation wavelength which is adjustable between about 240-500 nm, depending on the fluorescent species associated with the nucleic acid fragments, as discussed below. One exemplary fluorescence detector is an HP1046A detector available from Hewlett-Packard (Palo Alto, CA), and modified as above for capillary tube detection. The detector is connected to an integrator/plotter 45 for recording electrophoretic peaks.

Using the detector shown in Figure 2, the electro-osmotic flow rate measured during the FCSC method is determined by calculating the time required for a marker band to travel from the upstream end (end 22a) of the tube to the point in the tube, near the tube's downstream end, where the marker is seen by the detector. The electro-osmotic flow rate detected in this manner thus represents an average of the instataneous flow rates at the time the marker is introduced and at the time the marker is detected. It will be appreciated that the system can be modified to allow determination of an instantaneous flow rate, by placing directly upstream of the detector, a T-tube through which marker can be periodically introduced into the tube

In a typical FCSC method, the capillary tube is thoroughly washed, using the conditions described in Example 1, by drawing suitable cleaning and rinsing solutions through the tube by applying a vacuum to reservoir 32. The tube is then flushed with several volumes of the electrolytic buffer solution and a small volume, typically 1-10 nanoliters of sample material is loaded into the anodic tube end. A voltage is applied between the anodic and cathodic reservoirs, setting up an electro-osmotic flow within the tube, with introduction of

the compound for altering surface charge into end tube end.

Figure 2 shows a fragmentary view of an electrophoretic system 50 which can be operated in both a pulsed and constant-voltage mode. The capillary tube 52 in the system has a small-clearance break 54 adjacent and upstream of the detection zone, indicated at 56. The tube sections on either side of the break are coupled by porous glass sleeve 58 which allows electrolyte migration into and out of the tube. The coupled portion of the tube is sealed within a reservoir 60 filled with a suitable electrolyte solution 62. A grounded electrode 64 in the reservoir is connected to the high-voltage side of a pulsed-voltage power supply 66 whose negative side is in communication with a suitable cathodic reservoir. The grounded electrode is 64 is connected to the high-voltage side of a DC power supply 68 whose negative side is in communication with a suitable anodic reservoir.

II. <u>Electro-osmotic Flow</u>

This section describes the phenomenon of electro-osmotic flow. The phenomenon is exploited both in the FCSC method of the invention, for achieving a desired surface charge coating in the electrophoresis tubes, and in an electrophoetic separation of sample material, for optimizing electrophoretic separation between the sample species.

Figure 3 shows an enlarged, fragmentary portion of a capillary electrophoresis tube 70. As seen in the figure, the negatively charged groups on the inner tube wall, indicated by "-" symbols, are shielded by positively charged ions in the polymer solution, essentially forming a positively charged shell about the column of fluid in the tube. The thickness of the shell of relatively

immobilized positive ions at the wall surface is known as the shear distance. This outer shell of positive charge and inner bulk phase charge distribution is called an electric double layer, and is characterized by a zeta potential, which is a measure of the potential between the outer shell of positive charges and the bulk medium.

Under the influence of an electric field, this column of polymer solution in the medium (which is surrounded by a shell of positive charges) is drawn electro-osmotically in the direction of negative or low potential. The rate of electro-osmotic flow in the tube is indicated by the arrow e in the figure (arrow e may be thought of as a vector with a magnitude e and a direction along the axis of the tube). The electro-osmotic flow rate e in a capillary tube can be described by the equation:

$$e = \frac{\quad}{\quad}$$

where ε, , ξ, and *E* are the permittivity of the fluid, its viscosity, the zeta potential, and the electrical field strength, respectively.

The zeta potential, , as it applies to a charged wall surface, describes the potential across the interfacial double layer between the charged wall surface and the "inner surface" of the charged shell corresponding to the radius of shear of the shell. The potential is thus directly dependent on the net charge of the wall surface, and can be increased or decreased by increasing or decreasing the surface charge density, respectively, on the wall.

Figure 3 also illustrates how electrophoretic separation of sample species, such as the three nucleic acid

species shown at F_1, F_2, and F_3 in the figure, can be enhanced in a CE tube which is prepared, according to the present invention, to provide a selected electoosmotic flow rate. Here the rate of electro-osmotic flow is indicate by a vector e, indicating a magnitude e in the downstream direction in the figure. The three nucleic species migrate electrophoretically in the opposite direction at rates indicated by the vectors indicated at m_1, m_2, and m_3. The net rate of migration of each species in the tube is just the sum of the two opposing vectors, indicated by μ_1, μ_2, and μ_3, respectively.

It can be appreciated that the ability to separate the three species depends on the differences between the three migration-rate vectors μ_1, μ_2, and μ_3. These relative differences between these vectors, in turn, can be selectively controlled by varying e. For example, by making e close to m_3, the vector μ_3 can be made quite small and thus allow F_3 to be readily separated from the other two species. Likewise, by making e quite close to m_2, the vector μ_2 can be made quite small and thus allow F_2 to be readily separated from F_1.

The use of selected electrosmotic flow rate to achieve improved protein and nucleic acid separations will be described in more detail below.

III. <u>Flow-Rate Controlled Surface-Charge Coating (FCSC)</u>

The principle of FSCS is illustrated in Figure 4. The lines associated with the negative (-) and positive (+) symbols represent the electrodes. The line connecting the two reservoirs represents the capillary. T represents the amount of time required to traverse the length of the capillary and μ represents the electro-osmotic flow; the arrow adjacent μ represents the vector comprising the electro-osmotic flow. At T_0, for a given

applied voltage, the electro-osmotic flow is μ_0. When a positively substance is introduced into the anodic reservoir, for example a quaternary amine containing polymer (see below), and the voltage is applied, at some time T_i the electro-osmotic flow is decreased to μ_i (denoted as a shorter arrow). The magnitude of the charge on the capillary wall is directly related to electro-osmotic flow. The slowing in electro-osmotic flow with increased run time is the result of the coating of the capillary walls by the positively charged substance and the resulting net reduction in the charge of the capillary wall (Example 1; Figure 8). At some point in time, $T_{i+\Delta i}$, all the surface charges of the capillary will be neutralized and the electro-osmotic flow, $\mu_{i+\Delta i}$, will be zero.

A number of positively charged substances have been tested for their ability to effect flow-rate controlled surface charge coating. Figure 8 shows the effects of FCSC with spermine, is a non-polymeric primary amine. The reduction in electro-osmotic flow (V_{eo}) with increasing run time is apparent for the two concentrations shown (Example 3). Another important feature of these results is that the electro-osmotic flow plateaus fairly rapidly with increasing run time indicating that an equilibrium between the capillary wall bound spermine and the spermine in the buffer have reached an equilibrium. Maintenance of the reduced V_{eo} is dependent on the continued presence of spermine in the run buffer and the ionic strength of the buffer.

A second positively charged compound which affects V_{eo} is dodecyl trimethyl ammonium bromide (DTAB); a non-polymeric quaternary amine. The results of electrocoating with DTAB and the accompanying reduction in electro-osmotic flow (V_{eo}) with increasing run time is apparent for the concentration shown in Figure 9 (Example 4). As

can be seen from the figure, DTAB, as was seen with spermine, quickly reaches an equilibrium between the capillary wall bound DTAB and the free DTAB in the buffer.

Electrocoating with spermine and DTAB both have the limitation that calibration of the electro-osmotic flow using either compound provides a very narrow window for adjustment of the V_{eo}. A third positively charged substance used for electrocoating was hexadimethrin bromide (polybrene). The electrocoating data obtained with polybrene is shown in Figure 6 (Example 2) for the concentration of 0.0005%. Polybrene has two very important features which make it a good choice for an electrocoating agent: (1) as seen in Figure 6 the polybrene does not plateau (ie. establish an equilibrium) nearly as quickly as either spermine or DTAB; and (2) reversal of the polybrene coating requires an extensive wash cycle (see Example 1), thus it is not as sensitive as spermine to the ionic strength of the buffer. Polybrene does, however, have a range of stability depending on the electrophoresis buffer. To some degree the stability of the polybrene coating is dependent on the ion which is present in the buffer. Experiments performed in support of the present invention indicate that phosphate buffers are the most destabalizing and borate buffers achieve a greater stability.

Polymers used for electrocoating have two primary interactions with the capillary wall, ionic and hydrophobic. Accordingly, the characteristics of polymers useful for the adjustment of electro-osmotic flow in capillary electrophoresis include the following:

1. The polymer of choice should have multiple ionic binding centers. In the case of polybrene the binding centers are the quaternary amines.

2. The polymer should have some degree of hydrophobicity.

Polymers of the following form, $(NR_3)^{+}-(CH_2)_n-(NR_3)^{+}$, are particular suited to this application:

$$\left[\begin{array}{c} \quad\ R \qquad\qquad\quad R \\ \quad\ |{+} \qquad\qquad |{+} \\ -R-N-(CH_2)_n-N-R- \\ \quad\ | \qquad\qquad\quad | \\ \quad\ R \qquad\qquad\quad R \end{array}\right]_N ,$$

where R is a side group (such as hydrogen, an alkyl, aryl, or functional group) and N is the number of repeating units present in the polymer. Such polymers have a direct charge interaction between the quaternary amines and the negative charges on the wall and a hydrophobic interaction with the wall which results in further charge shielding. Polybrene is an example of one such polymer which masks charges on the capillary wall.

Example 2 describes the electrocoating of capillaries using two concentrations of polybrene; the effect of the electrocoating on electro-osmotic flow is represented in Figure 7. These data illustrate an important feature of the invention, in that, it can be seen that a precise relationship exists between electrocoating with polybrene for a designated time and the resulting electro-osmotic flow rate. Such a relationship is particularly valuable given polybrene's stable interaction with the capillary wall (as described above). A specific electro-osmotic flow can be chosen from a calibration curve for a given concentration of polybrene, as presented in Figure 7, and the run time to achieve that flow can be easily determined.

Another method of tuning electro-osmotic flow is the use of zwitterionic quaternary amines for electrocoating. The advantage of the zwitterionic compounds is that the V_{eo} is adjusted by completely coating the capillary sur-

face. This coating results in the neutralization of the charge inherent in the capillary surface and the substitution of the negative charge centers present in the zwitterionic compound. In other words, the only charge remaining contributing to the zeta potential are the negative charges present in the zwitterionic compound. For example, polybrene itself has multiple quaternary amine - positively charged centers, complete neutralization of the surface charge of the capillary with polybrene results in no net charge and V_{eo} decreases to zero. However, a polybrene-like polymer can be used for coating which has a charge group, such as carbonate or sulphonate, substituted in 50% of the R positions (see above). When the capillary is completely coated with this polymer, to the extent that all the inherent surface charges of the capillary wall are neutralized by the quaternary amine - positive-charge centers of the polymer, the net charge on the capillary wall will be approximately one-half of the original charge. The charge on the capillary wall is now the sole result of the negative-charge centers provided by the polymer and a new V_{eo} is established. The adjustment of electro-osmotic flow by such a coating process is calibrated by the negative-charge character of the coating polymer.

The ability to easily choose conditions to establish a specific electro-osmotic flow has valuable applications to research. For example, for any given separation application appropriate voltage and electro-osmotic flow conditions which maximize separation can be determined (eg. Examples 5-8) and then routinely and reproducible used to effect the separation.

Further, for repetitive separation applications, such as in a clinical setting, the capillary tube can be covalently modified to result in a fixed electro-osmotic

flow. This can be accomplished in one of two ways. First, the capillary tube can be coated with a polymer, such as polybrene, the exposed negatively-charged silane sites can be activated using a bifunctional reagent, then a second coating agent can be applied to the column and covalently attached to the capillary via the bifunctional reagent. Second, the selected flow rate can be established with the polymer, such as polybrene, and then the polymer covalently bound to the capillary by, for example, baking or dehydration by chemical means. Alternatively, the polybrene can be modified to contain a group which can be covalently coupled to the silane group of the capillary wall.

IV. <u>Applications to Protein Capillary Electrophoresis</u>

A. Overcoating to Block Interactions with the Capillary Wall.

A major limitation of the application of capillary electrophoresis to the separation of proteins is that many proteins have a net negative charge, the result of which is strong binding to the silane groups of the capillary wall. In this system using reduced pH to protonate the silane groups, and thus change the net charge of the wall, is not an acceptable option since a pH of less than 4 would be required and most proteins will not tolerate this pH.

The difficulty of separation of positively charged proteins by capillary electrophoresis is demonstrated by Example 5, Figure 10. Three isoforms of lactate dehydrogenase (LDH) derived from rabbit muscle was loaded onto a capillary under the conditions described in Example 5; LDH has a net positive charge. Figure 10 illustrates that there is no recovery of the loaded LDH. The most likely cause of the protein retention was the charge

interaction between the LDH and the capillary wall. In order to reverse the charge of the capillary wall the capillary was over-coated with polybrene.

Over-coating with polybrene is accomplished by electrocoating with polybrene at a selected concentration until the electro-osmotic flow, μ, decreases to zero. This condition occurs when the negative wall charges have been neutralized by an equivalent number of positive charges. But one property which makes polybrene and polymers of its kind particularly suitable for electro-coating, in addition to its charge interactions with the capillary wall, is the ability of the polymer to form hydrophobic interactions. These hydrophobic interactions occur with the capillary wall as well as with polybrene molecules which have already coated the capillary wall. After electro-osmotic flow is reduced to zero, further coating initially proceeds on the basis of electrostatic attraction of the cathode for the positively charged polymer. When the net charge of the capillary wall becomes positive, the electro-osmotic flow reverses itself and flows in the direction of the anode. In order to further coat the capillary, the polarity of the electrodes is reversed and the electro-osmotic flow is now able to draw more of the polybrene from the reservoir, through the capillary, resulting in overcoating and a net positive charge of the capillary wall.

Figure 11 illustrates the results of a capillary electrophoresis run performed under the same conditions as those used for Figure 10 but in this case the capillary was overcoated with polybrene. As can be seen from Figure 11 this capillary electrophoresis system, using the capillary over-coated with polybrene, provides an extremely efficient separation of the three isoforms of LDH.

Capillary electrophoresis was also performed using, as sample, the highly basic protein Histone H4 in five acetylated forms. Without over-coating of the capillary with polybrene these proteins do not traverse the capillary. On the other hand, when a polybrene over-coated capillary is used for the electrophoresis the system is capable of resolving the five acetylated forms of the protein (Example 5; Figure 12).

The ability to separate basic proteins in the capillary electrophoresis system provides an extreme valuable technique for the analysis of proteins, particularly when only small quantities of the proteins are available.

B. Capillary electrophoresis of proteins using selected electro-osmotic flow rates.

For negatively charged proteins no adverse charge interaction exists between the capillary wall and the protein. The separation of these proteins is based on a combination of electro-osmotic flow and charge attraction of the protein for the anode in the direction opposing electro-osmotic flow; accordingly, it is of great value to be able to adjust electro-osmotic flow to tune protein separation. As described above, calibration curves derived for a given polymer concentration are useful tools for the determination of the conditions necessary to achieve a selected V_{eo}.

One example of the use of partial coating to achieve separation of a negatively charged protein is shown in Figure 13 (Example 6). The capillary was pre-coated for 5 minutes using 0.001% polybrene followed by loading 2.5 ng of an equal-part mixture of Ribonuclease T1 (RNase T1), which has a net negative charge, and a recombinantly created mutant species having a glutamine to lysine substitution. This substitution in the mutant species

results in it having one more positive charge than the wild type species. Figure 13 clearly demonstrates the remarkable separation achieved using this method. The neutral marker is represented by peak 6.67 and the two species of RNase T1 by peaks 14.26 and 15.96.

C. Ion-exchange Capillary Electrophoresis of Negatively-Charged Proteins.

Another type of separation which can be achieved using partial coating involves a competition between salt and the charged protein for charge centers on the capillary wall. This competition is illustrated for RNase T1 and a mutant species (described above) in Figures 17 and 18. The capillary was partial coated with polybrene and the sample mixture of RNase T1 species was loaded on the capillary using 10 mM sodium citrate buffer (pH=6.6) for the running buffer (Example 8). The electropherogram resulting from this run is shown in Figure 17; in this figure there are no peaks corresponding to either RNase T1 species. The RNase species are, most likely, held up in the column as a result of their charge interaction with the polybrene. When the capillary was washed and the partially coated capillary was loaded with the two RNase T1 species in the same buffer plus 10 mM sodium chloride peaks corresponding to both species were detected. The same method was repeated using increasing concentrations of sodium chloride. The maximum yield of the RNase T1 proteins was obtained with 30 mM NaCl (Figure 18).

This ability to selectively affect the retention of negatively charged proteins has useful applications to the separation of proteins having differing ionic character. Given a mixture of proteins having differing ionic character, conditions can be chosen which result in the

retention of one or more protein(s) and allows other proteins of the mixture to migrate through the capillary and undergo capillary electrophoretic separation. A further application of this technology is the concentration and subsequent separation of negatively-charged proteins; this application of the capillary electrophoresis system is described below in relation to nucleic acid separation.

V. Applications to Nucleic Acid Capillary Electrophoresis

For the separation of nucleic acids an neutral-polymer is added to the electrophoresis buffer which establishes a fluid fractionation matrix in the capillary tube. Nucleic acid separation methods and further applications based on the inclusion of neutral polymers in the electrophoresis buffer are disclosed in co-owned U.S. Patent Application No. 390,631.

Counter-migration capillary electrophoresis (CMCE) can be illustrated by the migration of negatively charged nucleic acids. At the same time the electrolytic solution in the capillary tube is moving downstream (toward the cathodic reservoir) by electro-osmotic flow, the negatively charged nucleic acid fragments are migrating relative to the solution in the opposite direction toward the anodic reservoir. Because of molecular interactions of the fragments with the neutral-polymer molecules, the rates of fragment migration toward the anodic reservoir are size dependent, with smaller fragments migrating faster in the anodic direction.

Capillary electrophoretic separation of nucleic acids is dependent on electro-osmotic flow as is the separation of proteins; the ability to adjust V_{eo}, as described for proteins, is also a valuable asset for the

separation of these macromolecules. One useful approach for adjusting V_{eo} for the resolution of nucleic acids is coating the capillary with a zwitterionic compound (see above). Using zwitterionic compounds a V_{eo} can be selected and charge-repulsion between the nucleic acids and the capillary wall can be maintained.

Another important application of capillary electrophoresis to the separation of nucleic acids is the ion exchange phenomena which was discussed above for negatively charged protein. Example 7 describes the partial coating of a capillary with polybrene and the effects on nucleic acid separation. When a DNA sample was run in SB buffer in the absence of salt no peaks corresponding to the loaded DNA sample were detected (Figure 14). However, when the same sample was run in the presence of 10 mM NaCl several DNA species were resolved (Figure 15); further DNA species were resolved when the salt concentration was increased to 20 mM NaCl (Figure 16). As for negatively-charged proteins it appears that the salt is competing for binding to the polybrene resulting in a general ion exchange phenomenon.

The ion exchange phenomenon can be exploited for separation of nucleic acids. Given a mixture of nucleic acid species conditions can be established such that the larger molecules in the mixture may be bound to the capillary wall allowing more efficient resolution of smaller species in the mixture.

Another important application of the ion exchange phenomenon is the ability to concentrate dilute solutions of biologically important macromolecules; this application is important to many clinical and research problems. To concentrate, for example, a dilute sample of nucleic acid, a small portion of the capillary tube is coated

with polybrene to result in a net positive charge for the coated region. The dilute sample is then loaded onto the capillary and voltage applied. The nucleic acids are arrested and accumulate in the region of positive charge by virtue of their ionic interactions with the region. The capillary is then treated with a running buffer of sufficient ionic strength to cause release of the nucleic acid. The release occurs in a small zone at the front of the high salt buffer. The released nucleic acids are then resolved by counter-migration capillary electrophoresis, as described above, over the rest of the length of the capillary. This method of concentrating macromolecules can be applied to negatively-charged proteins as well.

The following examples illustrate various separation methods and applications in accordance with the invention, but are in no way intended to limit the scope thereof.

Example 1

Changes in Electro-osmotic Flow as a Function of Polybrene Coating Llevel

Capillary electrophoresis was carried out using an ABI Model 270 Capillary Electrophoresis System. The system includes a built-in high-voltage DC power supply capable of voltage settings up to 30 KV. The capillary tube used in the system is a fused silica capillary tube 72 cm long with a 50 µm i.d. and 350 µm o.d.obtained from Polymicro Technologies (Phoenix, AZ).

The marker used to indicate the rate of electro-osmotic flow (V_{eo}) was the neutral compound, mesityl oxide, which has a strong absorbance at 200 mm. The electrophoretic system was run at a voltage setting of about 25 kV (about 350 V/cm) through the run. UV detec-

tion was with a Kratos 783 UV detector designed for capillary tube detection. The detector output signal was integrated and plotted on a Spectrophysics Sp4400 integrator/plotter.

Fresh capillary surface was routinely regenerated by flushing the capillary successively with 5-10 capillary volumes of 1.0 N NaOH, 3-5 volumes H_2O, 3-5 volumes 0.1 N HCl, 3-5 volumes H_2O, and finally 3-5 volumes of 5 mM $NaPO_4$ (pH 7.0) buffer. Solutions are drawn through the capillary by virtue of vacuum applied at the cathodic end.

In general, after equilibration with the approximate buffer, 2-5 nl of neutral marker (mesityl oxide) is injected into the capillary by virtue of vacuum applied at the cathodic end; the marker is used to measure electro-osmotic flow. Injection of the marker was followed by injection of 2-5 nl (2-10 ng) of protein sample; either marker or sample may be omitted from the cycle. Appropriate voltage (up to 30 kV) may then be applied across the ends of the capillary and separation monitored via the UV detector. Electrocoating was accomplished by the application of voltage from the anodic reservoir containing buffer plus polybrene (hexadimethrin bromide; available from Applied Biosystems, Foster City, CA) to the cathodic reservoir containing buffer with no polybrene.

Successive injections of marker (2.5 nl) into the capillary followed by one minute pulses of 25 kV result in the migration of the successive marker peaks and anodic buffer with polybrene through the capillary and eventually past the detector. Absorbance at 200 nm was monitored and the average velocity of marker, and therefore the electro-osmotic flow, was estimated. There was no detectable absorbance of polybrene under these conditions.

Total run time was about 70 minutes the final approximately 50 minutes are shown in Figure 5; minutes 20 to 70 are noted at five minute intervals at the bottom of the electropherogram. The concentration of polybrene in the anodic reservoir was 0.0005%. At approximately 24 minutes the polybrene had migrated the entire length of the capillary tube. The slowing of the electro-osmotic flow rate is apparent from the increasing distance between the neutral marker peaks which occurs as the run progresses and the capillary becomes progressively more coated with the polybrene.

Example 2

Calibrating electro-osmotic flow rates

The same capillary electrophoretic conditions used in Example 1 were employed. The rate of electro-osmotic flow (V_{eo} represented in cm/min) was calculated based on the distance traversed and the time elapsed from injection of the neutral marker until the pulse passed the UV detector. The calculated V_{eo} was plotted against the total elapsed run time; the results are represented in Figure 6. Polybrene at a concentration of 0.0005% was present in the anionic reservoir at time 0.

Electrocoating using polybrene at a concentration of 0.001% was also performed. The Ln of (V_{eo} + 1) was plotted against the total elapsed run time for the polybrene concentrations of 0.0005% and 0.001% (Figure 7). As can be seen from Figure 7 at these polybrene concentrations a reliable relationship is established between coating time, the concentration of the polymer and the electro-osmotic flow rate. For example, the same V_{eo} can be established by coating with 0.001% polybrene for approximately 25 minutes versus coating with 0.0005% polybrene for approximately 42 minutes.

Example 3
Calibrating Electro-osmotic Flow Rate
Using a Non-Polymeric Primary Amine

The same capillary electrophoretic conditions used in Example 1 were employed and V_{eo} was calculated as above. spermine, a non-polymeric primary amine, was used as the coating agent at concentrations of 0.001% and 0.005% and its affects on V_{eo} with increasing run time are shown in Figure 8. As can be seen from Figure 8 the V_{eo} plateaus rapidly with spermine relative to polybrene (Figure 6); this results in less sensitivity for calibration of V_{eo} with spermine. A further limitation of spermine is that it can be removed by ions in the electrophoresis buffer.

Example 4
Calibrating Electro-osmotic Flow Rate
Using a Non-Polymeric Quaternary Amine

The same capillary electrophoretic conditions used in Example 1 were employed and V_{eo} was calculated as above.

Dodecyl trimethyl ammonium bromide (DTAB), a non-polymeric quaternary amine, was used as the coating agent at a concentration of 0.15% and the effects on V_{eo} are represented in Figure 9. DTAB has one of the same limitations as spermine, in that, relative to polybrene DTAB plateaus rapidly, thus calibration of V_{eo} using DTAB is not as sensitive as polybrene.

Example 5
CE Electrophoresis of Proteins

A. Lactate Dehydrogenase

Capillary electrophoresis was performed as described in Example 1. Approximately 2.5 ng of lactate dehydrogenase (LDH) from rabbit muscle containing three isoforms (pI = 8.3, 8.4, 8.55) was loaded in 5 mM $NaPO_4$ buffer (pH=7.0) with the addition of 2.5 nl of the neutral marker. In the absence of polybrene coating only the neutral marker was seen to flow through the column. Figure 10 shows the results of the run in the absence of polybrene coating of the capillary; the single peak at 3.10 mins. is the neutral marker.

Figure 11 shows the results of a similar run where the capillary tube has been pre-coated using 0.01% polybrene for 10 minutes; this degree of pre-coating results in over-coating of the capillary tube, reversal of the negative charge on the capillary surface, and the concomitant reversal of direction of electro-osmotic flow. The polarity of the electrodes was reversed before loading of the protein sample. No polybrene was present in the electrophoresis buffer and 2.5 ng of LDH isoforms were loaded. The results of the LDH run in the presence of polybrene overcoating is shown in Figure 11. The peaks at 17.82, 18.19, and 20.32 correspond to the 8.3, 8.4 and 8.55 isoforms, respectively. The shoulder at 19.68 represents a contaminant of the preparation, probably formed as the result of storage of the LDH. As can be seen from comparison of Figures 10 and 11 effective separation can only be achieved with LDH when the capillary has been coated with polybrene.

B. Acetylated Histone H4

Capillary electrophoresis was performed as described in Example 1. The capillary was over-coated with polybrene as above to establish an electro-osmotic flow rate of 5.51 cm/min. The polarity of the electrodes was reversed before loading the proteins. The electrophoresis buffer was 10 mM Na-citrate, pH=6.6; the running buffer did not contain additional polybrene. Approximately 3.0 ng of multiply acetylated histone H4's, containing 0-4 acetyl groups, was loaded for the run. Figure 12 shows the ability of the system to resolve the five acetylated forms of H4. The proteins corresponding to the peaks shown in the electropherogram are as follows (peak/number of acetyl groups): 9.01/0; 8.87/1; 8.64/2; 8.4/3; and, 8.19/4. Without coating these highly basic proteins do not traverse the capillary but remain attracted to the capillary surface (data not shown).

Example 6

Protein Separation by CE as a Function of Electro-osmotic Flow Rate

A. Capillary tube preparation for selected electro-osmotic flow rates.

The capillary tubes were prepared by pre-coating the capillary for 5 min using 0.001% polybrene in 5 mM Na-PO_4 (pH 7.0) buffer.

B. Separation of two species of Ribonuclease T1

Capillary electrophoresis was performed as described in Example 1. The pre-coated capillary described in section A was used. The running buffer was 5 mM Na-PO_4 (pH=7) with the addition of 20 mM NaCl; no additional polybrene was added to the running buffer. An equal part

mixture was prepared of wild-type <u>Aspergillus oryz</u> Ribonuclease T1 (RNase T1) and a recombinant mutant species having a glutamine to lysine substitution; this substitution results in the mutant having one more positive charge than wild-type. Approximately 2.5 ng of the protein mixture was loaded and separation carried out for 16 minutes at 25 kV. The neutral marker is represented by the peak at 6.67. No loss in coating is observed under these conditions.

The exquisite sensitivity of this seperation system can be seen in the two well defined peaks (14.26 and 15.96; Figure 13) corresponding to the two species of RNase T1. The very sharp spikes are correspond to precipitates formed in the running buffer; this precipitate can be eliminated by pre-filtration, but its presence does not affect separation.

Example 7

Constant Field Counter-migration Capillary Electrophoresis (CMCE) of Deoxyribonucleic acid in 0.15% Hydroxyethyl Cellulose using Polybrene coated capillaries.

Capillary electrophoresis was performed as in Example 1 except the capillary was electrocoated with polybrene before loading of the DNA sample and the buffer was SB (5 mM Na-borate (pH=9) containing 0.15% hydroxyethyl cellulose).

The extent of electrocoating was monitored as described in Example 1. Electrocoating was allowed to proceed for five minutes in SB, with polybrene at a concentration of 0.001%, which under these conditions resulted in a decrease in endoosmotic velocity of 29% (from 17.0 cm/min to 13.1 cm/min). DNA peaks were monitored at 260 nm.

Three nanograms of DNA (1kb ladder obtained from Bethesda Research Lab.) was loaded onto the column and in the course of a 30 minute run no DNA peaks were detected (Figure 14).

The capillary was then washed with the SB buffer plus 10 mM NaCl. DNA was loaded and run as above, using SB + 10 mM NaCl as running buffer. As can be seen from Figure 15, several DNA peaks were then detected during the 30-min run. The capillary was washed with the SB buffer plus 20 mM NaCl. DNA was loaded and run as above, using SB + 20 mM NaCl as running buffer; more DNA species were detected (Figure 16). It is apparent from comparing these two figures that specific DNA species can be released in an ion exchange fashion using, in this case, a step gradient. Figures 15 and 16 show that peaks are sharp and the DNA fragments are well resolved.

Example 8

Ion-Exchange Capillar Electrophoresis (IECE) of Anionic Proteins

Partial coating of the capillary was achieved with 10 mM Na-Citrate buffer (pH 6.6) essentially as described in Example 7. Ribonuclease T1 (pI=2.9) and a mutant species of RNase T1, differing by one positive charge (see Example 6), were loaded onto a capillary, under vacuum. The capillary electrophoretic conditions were as described in Example 1. Figure 17 shows the results of the run; the peak at 4.82 is the neutral marker and the spike at 14.98 indicates the end of the run. There is an absence of a definable peak at 200 nm which would correlate to RNase T1.

At pH=6.6 RNase T1 the mutant T1 are negatively charged. When the capillary was washed with the 10 mM Na-citrate buffer + 10 mM NaCl and more protein was

applied two peaks appeared. Recovery increased as the same amount of protein was loaded with increasing salt concentration until a maximum yield of protein was obtained at 30 mM NaCl (Figure 18).

While the invention has been described with repect to specific embodiments, methods, and applications, it will be recognized by those skilled in the art that modifications of the methods and application of the methods to other uses involving the separation of macromolecules may be made without departing from the invention.

IT IS CLAIMED:

1. A method of achieving selected electro-osmotic flow characteristics in a capillary tube having charged surface groups, comprising

placing opposite ends of the tube in anodic and cathodic electrolyte reservoirs,

applying an electric field across the two reservoirs to produce electroosomotic flow from one tube end toward the other tube end,

drawing into and through the tube, a compound capable of altering the surface charge of the tube, as the compound is drawn through the tube,

monitoring the electro-osmotic flow rate within the tube as the compound is drawn into and through the tube, and

continuing to draw said compound into and through the tube until a desired electro-osmotic flow rate in the tube, as determined from said monitoring, is achieved.

2. The method of claim 1, wherein said monitoring includes introducing into said tube, at spaced time intervals, a plurality of pulses of a flow marker whose travel through the tube can be used to monitor the electro-osmotic flow rate of a band of fluid in the tube containing the marker solution.

3. The method of claim 1, wherein said tube is a quartz glass tube having negatively charged surface silane groups, and said compound is a polymer containing regularly spaced, charged amine groups.

4. The method of claim 2, wherein the the polymer is a quaternary amine, hydrophobic polymer.

5. The method of claim 4, wherein the polymer is selected from the group of polymers of the form

$N(R_3)^+-(CH_2)_n-N(R_3)^+$, where n = 2=10, where R is H or an alkyl or aryl group.

6. The method of claim 5, wherein the polymer is polybrene.

7. The method of claim 1, wherein the tube is a quartz glass tube having positively charged amine groups, and said compound is a negatively charged polymer selected from the group consisting of a polysulfonic acid, polycarboxylic acid, polyphorponic acid, and polyphosphoric acid polymer.

8. The method of claim 1, wherein the surface charge groups are due in part to molecules of a charged coating agent which is bound to the surface walls electrostatically, and said compound is effective to promote removal of the coating agent molecules from the surface walls, ass the compound is drawn through the tube.

9. The method of claim 1, wherein the compound is a charged polymer compound which is effective to bind to the surface walls of the tube electrostaitcally and to itself hydrophobically, as the compound is drawn into and through the tube, said drawing includes drawing the compound into the tube in the initial direction of electro-osmotic flow, until the charge on the surface walls is neutralized and electro-osmotic flow in said initial direction ceases, and further drawing the charged compound through the tube in the same direction until electro-osmotic flow within the tube, in the opposite direction, reaches a selected rate.

10. The method of claim 9, wherein said capillary tube walls have negative charges, and the charged polymer compound is a hydrophobic quaternary amine polymer.

11. The method of claim 1, for use in preparing a capillary electrophoresis tube for use in separating at least two macromolecular species having different size and/or charge properties in a selected electrophoresis medium, wherein the electro-osmotic flow characteristics are selected to enhance the degree of separation between the macromolecules which can be achieved by capillary electrophoresis in the selected medium.

12. The method of claim 11, for use in separating positively charged proteins, wherein said surface walls have negatively charged groups, and said compound is a hydrophobic polyamine polymer which is effective to bind to the surface walls of the tube electrostatically and to itself hydrophobically, as the compound is drawn into and through the tube, said drawing includes drawing the compound into the tube in the initial direction of electro-osmotic flow, until the negative charge on the surface walls is at least substantially neutralized.

13. The method of claim 12, wherein said drawing is continued until electro-osmotic flow in the initial direction ceases, and further includes drawing the charged compound through the tube in said initial direction until electro-osmotic flow within the tube, in the opposite direction, reaches a selected rate.

14. The method of claim 11, for use in separating nucleic acid species, wherein said surface walls have negatively charged groups, and said compound is a hydrophobic polyamine polymer, which further includes electrophoretically separating the nucleic acid species in an electrophoretic medium whose ionic strength at which at least one of the species to be separated does not bind to the polymer compound on the surface walls.

15. The method of claim 14, wherein the ionic strength of the electrophoretic medium is such as to cause preferential binding of the different nucelic acid species to the polymer bound to the capillary surface walls.

16. The method of claim 1, which further includes attaching the compound to the walls, by dehydrating by baking or chemical means.

17. The method of claim 1, for use in producing a capillary tube having a selected density of covalently attached charged groups on the surface of the capillary wall, wherein said compound contains chemical groups which can be covalently linked to surface wall groups, which further includes coupling the compound covalently to the surface walls.

18. A capillary tube having charged surface groups and selected electro-osmotic flow characteristics, prepared by the steps of:

placing opposite ends of the tube in anodic and cathodic electrolyte reservoirs,

applying an electric field across the two reservoirs to produce electroosmotic flow from one tube end toward

applying an electric field across the two reservoirs to produce electroosomotic flow from one tube end toward the other tube end,

drawing into and through the tube, a compound capable of altering the surface charge of the tube, as the compound is drawn through the tube,

monitoring the electro-osmotic flow rate within the tube as the compound is drawn into and through the tube, and

continuing to draw said compound into and through the tube until a desired electro-osmotic flow rate in the tube, as determined from said monitoring, is achieved.

19. The tube of claim 18, wherein said tube is a quartz glass tube having negatively charged surface silane groups, and said compound is a polymer containing regularly spaced, charged amine groups.

20. The tube of claim 19, wherein the the polymer is a quaternary amine, hydrophobic polymer.

AMENDED CLAIMS
[received by the International Bureau
on 22 April 1991 (22.04.91);
original claims 1-20 replaced by amended claims 1-20 (5 pages)]

1. A method of separating macromolecules in a capillary electrophoresis tube whose inner wall has charged surface chemical groups, said method comprising

drawing into a capillary tube, a solution containing a polyionic polymer having repeating subunits with charged chemical groups whose charge is opposite to that of the chemical groups of the tube's inner wall,

by said drawing, binding the polyionic polymer to the tube's inner wall by non-covalent chemical group interactions with a binding stability sufficient to retain the polymer on the tube wall during electroosmotic movement through the tube of an electrolyte which does not contain the polymer,

continuing said drawing until the tube's surface charged groups are substantially masked by the charge on the polymer,

immersing the ends of the tube in anodic and cathodic reservoirs containing an electrolyte solution,

introducing a sample containing macromolecules to be separated into one end of the tube,

applying an electric field across the reservoirs with a polarity effective to draw macromolecules in the sample from the one tube end toward the opposite tube end.

2. The method of claim 1, wherein said tube has anionic surface chemical groups, and the repeating subunits in the polymer contain cationic chemical groups.

3. The method of claim 2, wherein said anionic surface groups are silane groups, and said polymer contains regularly spaced, charged amine groups.

4. The method of claim 2, wherein said polymer is a hydrophobic polymer with quaternary amine charged groups.

5. The method of claim 2, wherein the polymer is selected from the group of polymers of the form

$[-N^+(R_3)-(CH_2)_n-N^+(R_3)-]_m$, where R is a side group (such as hydrogen, an alkyl, aryl or functional group), n = 2-10, and m is the number of repeating units present in the polymer.

6. The method of claim 4, wherein the polymer is hexamethrine bromide.

7. The method of claim 1, wherein the polymer is capable of forming hydrophobic intermolecular interactions and the solution containing the polymer additionally contains an agent capable of decreasing such hydrophobic interactions.

8. The method of claim 7 wherein the agent is ethylene glycol.

9. The method of claim 1, which further includes, following said applying, drawing through the tube a solution effective to remove the polymer from the tube walls.

10. The method of claim 1, for use in separating polypeptides, wherein said bound polymer confers a net positive charge to the tube's inner walls and said electrolyte in the tube during said applying has a pH below the isoelectric point of the polypeptides.

11. The method of claim 1, wherein the tube has positively charged amine groups, and said polymer is a negatively charged polymer selected from the group consist-

ing of a polysulfonic acid, polycarboxylic acid, and polyphosphoric acid polymer.

12. The method of claim 11, for use in separating nucleic acid species.

13. The method of claim 1, wherein the polymer is a zwitterionic polymer.

14. A method of achieving selected electro-osmotic flow characteristics in a capillary tube having charged surface groups, comprising

placing opposite ends of the tube in anodic and cathodic electrolyte reservoirs,

applying an electric field across the two reservoirs to produce electro-osmotic flow from one tube end toward the other tube end,

drawing into and through the tube, a compound capable of altering the surface charge of the tube, as the compound is drawn through the tube,

monitoring the electro-osmotic flow rate within the tube as the compound is drawn into and through the tube, and

continuing to draw said compound into and through the tube until a desired electro-osmotic flow rate in the tube, as determined from said monitoring, is achieved.

15. The method of claim 14, wherein said tube has anionic surface chemical groups, and said compound is a polymer containing regularly spaced, cationic chemical groups.

16. The method of claim 15, wherein the polymer is selected from the group of polymers of the form

$[-N^+(R_3)-(CH_2)_n-N^+(R_3)-]_m$, where R is a side group (such as hydrogen, an alkyl, aryl or functional group), n = 2-10, and m is the number of repeating units present in the polymer.

17. The method of claim 14, wherein the charged surface groups are due in part to molecules of a charged coating agent which is bound to the surface walls electrostatically, and said compound is effective to promote removal of the coating agent molecules from the surface walls, as the compound is drawn through the tube.

18. The method of claim 14, wherein the compound is a charged polymer compound which is effective to bind to the surface walls of the tube electrostatically and to itself hydrophobically, as the compound is drawn into and through the tube, said drawing includes drawing the compound into the tube in the initial direction of electro-osmotic flow, until the charge on the surface walls is neutralized and electro-osmotic flow in said initial direction ceases, and further drawing the charged polymer compound through the tube in the same direction until electro-osmotic flow within the tube, in the opposite direction, reaches a selected rate.

19. The method of claim 14, for use in separating nucleic acid species, wherein said capillary surface walls have negatively charged groups, and said compound is a hydrophobic polyamine polymer, which further includes electrophoretically separating the nucleic acid species in an electrophoretic medium whose ionic strength at which at least one of the species to be separated does not bind to the polymer compound on the capillary surface walls.

20. A capillary tube having charged surface groups and selected electro-osmotic flow characteristics, prepared by the steps of:

placing opposite ends of the tube in anodic and cathodic electrolyte reservoirs,

applying an electric field across the two reservoirs to produce electro-osmotic flow from one tube end toward the other tube end,

drawing into and through the tube, a compound capable of altering the surface charge of the tube, as the compound is drawn through the tube,

monitoring the electro-osmotic flow rate within the tube as the compound is drawn into and through the tube, and

continuing to draw said compound into and through the tube until a desired electro-osmotic flow rate in the tube, as determined from said monitoring, is achieved.

STATEMENT UNDER ARTICLE 19

The amended claims retain the two independent claims included in the original filing as new claims 14 and 20. New dependent claim 15 recites the limitation that the capillary tube used in the method of claim 14 has anionic surface chemical groups and that the compound drawn through the tube is a polymer having regularly spaced cationic chemical groups. This embodiment of the invention is supported in the specification at page 8, line 22 - page 9, line 2 and page 16, line 27 - page 17, line 18.

The polymeric formula of new claim 5 and 16, which was originally in claim 5, has been modified to represent the polymeric aspect of the compound. Support for the generalized nature of a polymer useful in the method of the invention is found in the specification on page 16, line 27 to page 17, line 18.

New independent claim 1, and dependent claims 2-13 are directed at an aspect of the invention which is generally described in the specification. The invention defines a method for separating macromolecules, particularly polypeptides and nucleic acids. Claim 1 recites several elements of the invention which are particularly useful in this method, and which also distinguish it from the prior art. One such element is the use of a polyionic polymer which binds non-covalently to the inner surface of the tube. This non-covalent coating permits titration of the amount of coating producing a charge environment which is optimal for a given separation, as mentioned on page 17, lines 22-32 of the specification. In addition, the non-covalent nature of the coating renders it reversible, facilitating repeated uses of the same capillary tube under different coating conditions.

These latter aspects of the invention particularly distinguish it from U.S. Patent No. 4,680,201 to Hjerten and U.S. Patent No. 4,865,706 to Karger, cited in the International Search Report. The coatings described in these patents are covalently bound to the inner wall of the capillary, either directly or through a bifunctional reagent. In addition, the coating of the invention described by Hjerten is directed to inhibition of electroendosmotic (electro-osmotic) flow; whereas embodiments of the present invention exploit this type of flow to provide improved separation characteristics in a capillary tube.

U.S. Patent No. 4,865,707 to Karger describes a gel-containing microcapillary tube, for use in gel electrophoresis. The gel is preferably covalently bound to the inner wall of the capillary tube through a bifunctional reagent which covalently attaches to both the inner wall of the tube and to the gel. In a described embodiment, the bifunctional reagent coating is adsorbed, rather than covalently bound, to the inner wall surface; however, this embodiment particularly refers to tubes composed of inert, uncharged materials, such as Teflon®. The gel contents of the tube are covalently bound to the reagent coating of the tube. In the present invention, the use of tubes having charged inner surfaces is preferred, and a charged coating binds to the charged inner surface of the tube through non-covalent charge-charge interactions. Furthermore, the contents of the tube as it is used during operation are not covalently attached to the coating material, so that variable electrophoretic media can be employed.

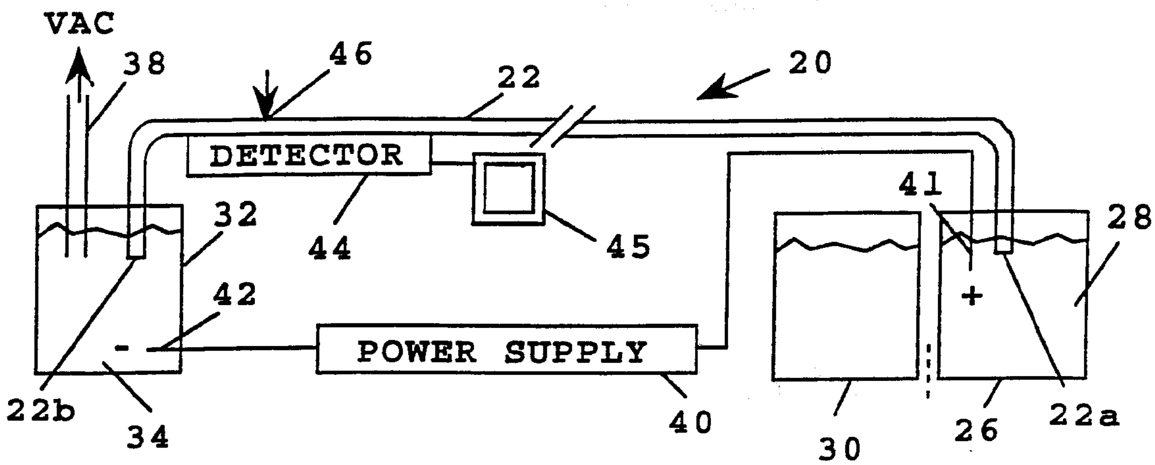


Fig. 1

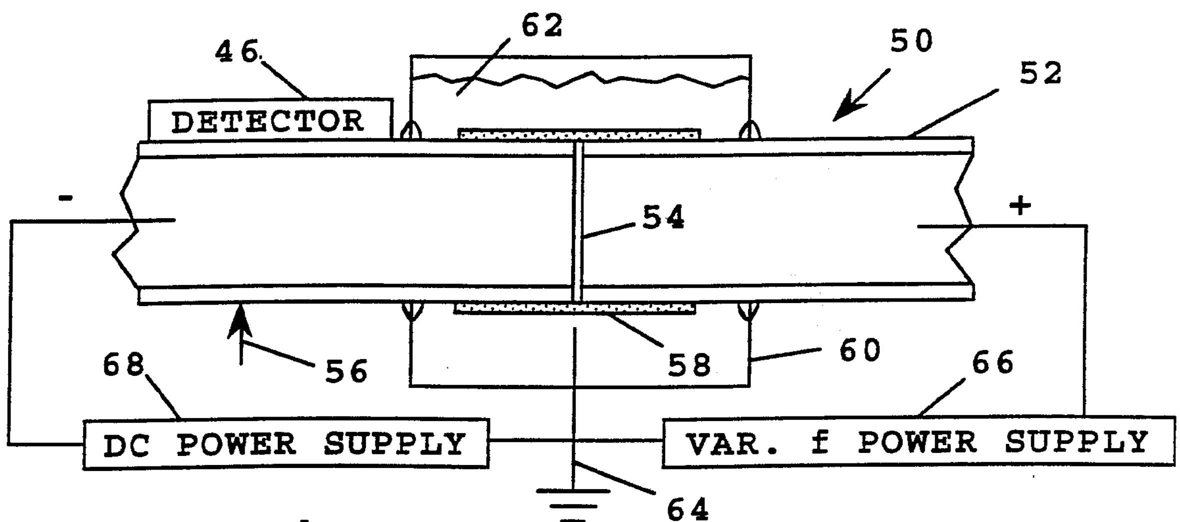


Fig. 2

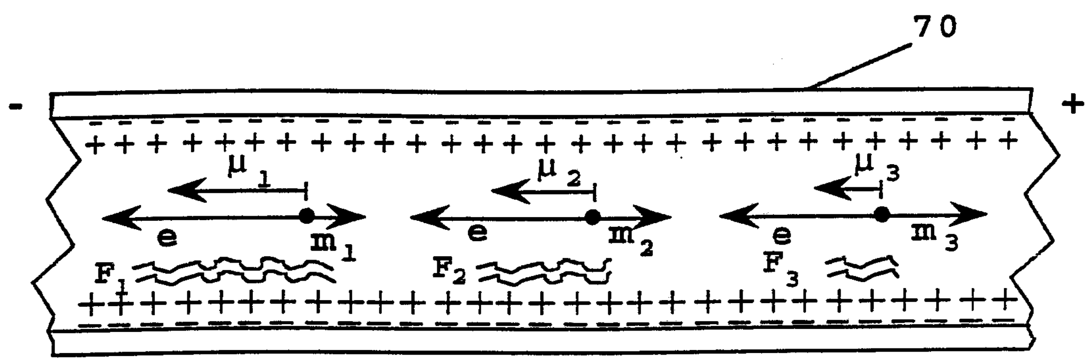


Fig. 3

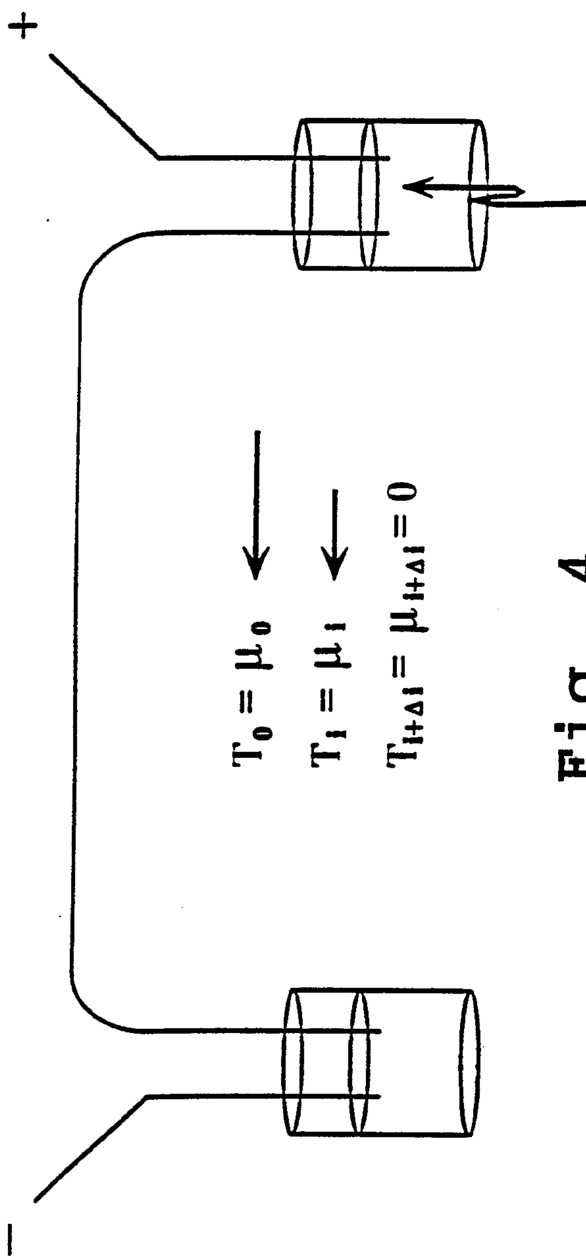


Fig. 4

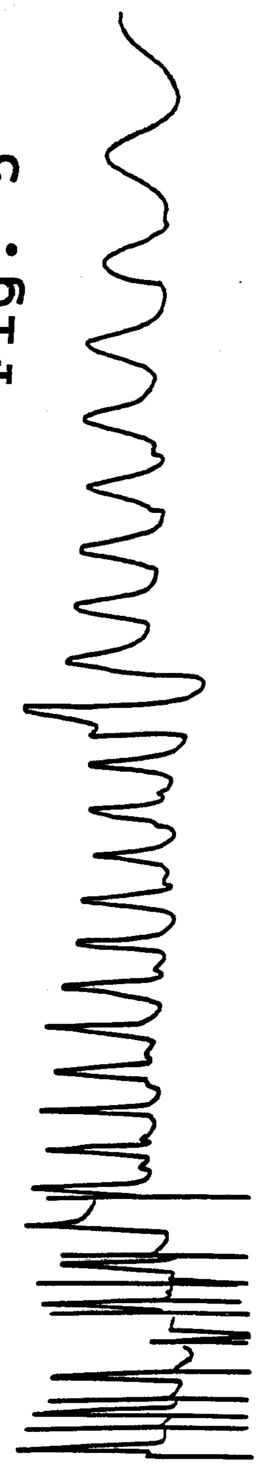


Fig. 5

3/8

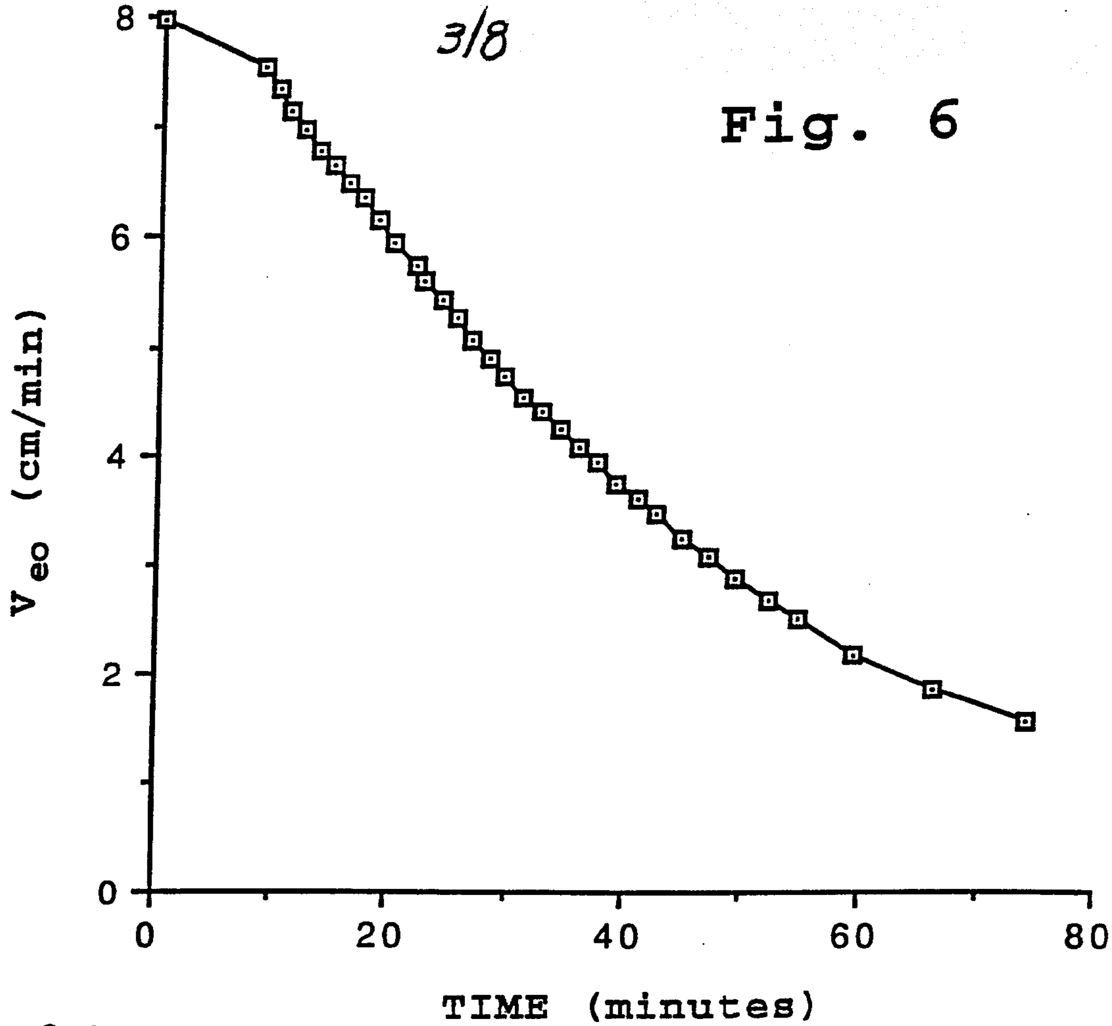

8
6
4
2
0
0
20
40
60
80
V_{eo} (cm/min)
TIME (minutes)

Fig. 6

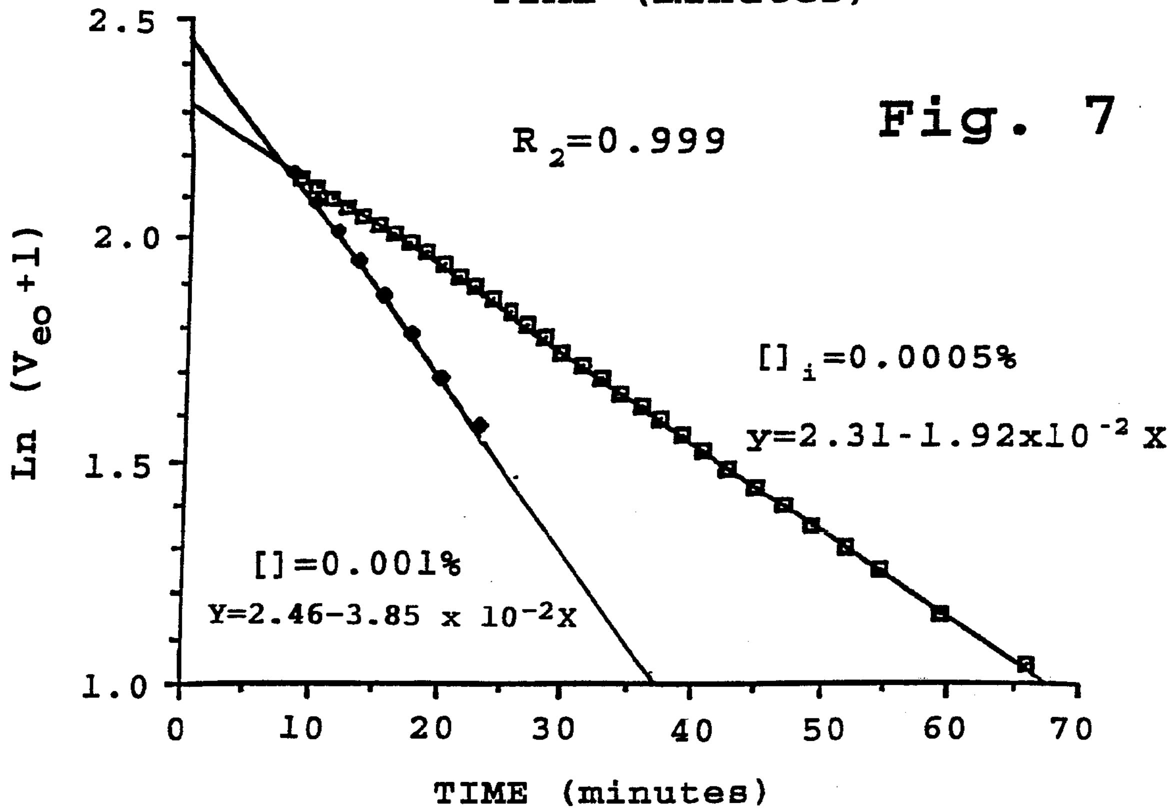

2.5
2.0
1.5
1.0
0
10
20
30
40
50
60
70
R_2=0.999
[]$_i$=0.0005%
y=2.31-1.92x10^{-2} X
[]=0.001%
Y=2.46-3.85 x 10^{-2}X
Ln (V_{eo}+1)
TIME (minutes)

Fig. 7

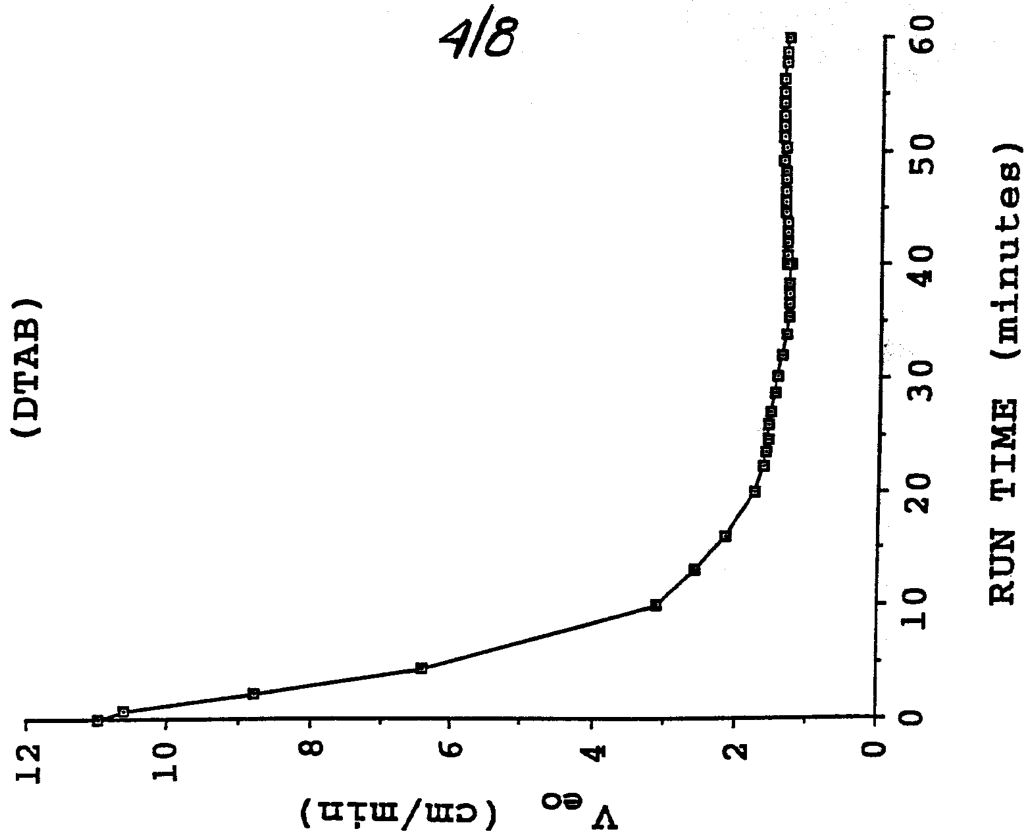

(DTAB)
V eo (cm/min)
12
10
8
6
4
2
0
0
10
20
30
40
50
60
RUN TIME (minutes)

Fig. 9

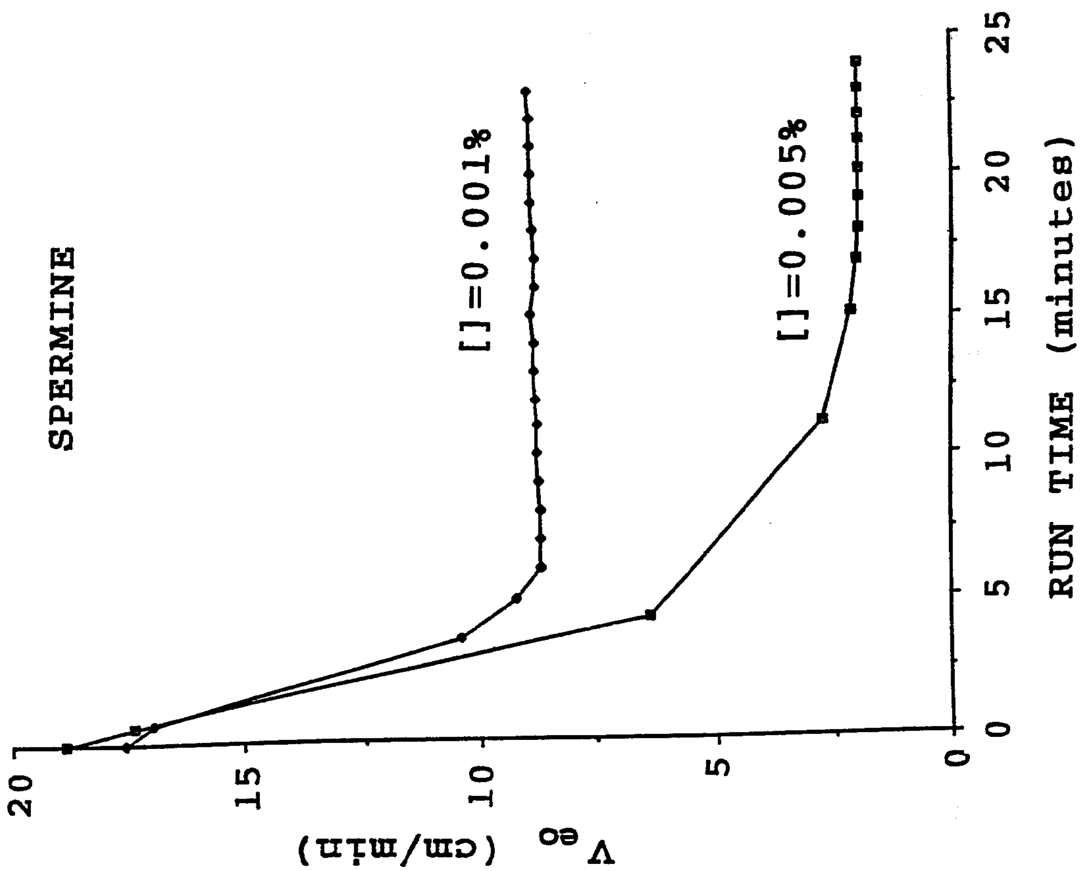

SPERMINE
[]=0.001%
[]=0.005%
V eo (cm/min)
20
15
10
5
0
0
5
10
15
20
25
RUN TIME (minutes)

Fig. 8

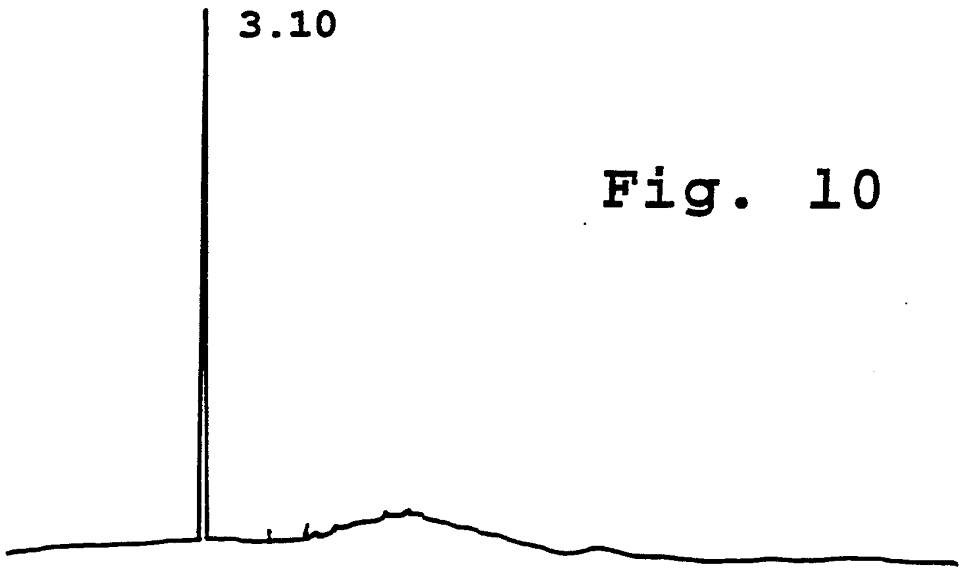


Fig. 10

Fig. 11

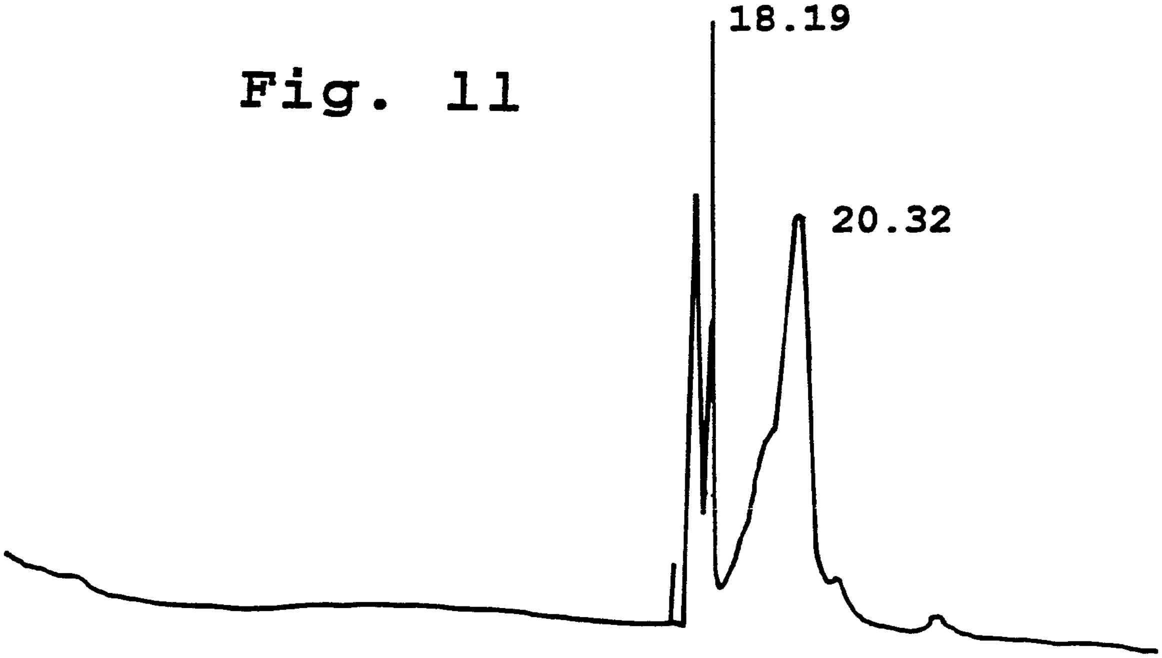


Fig. 12

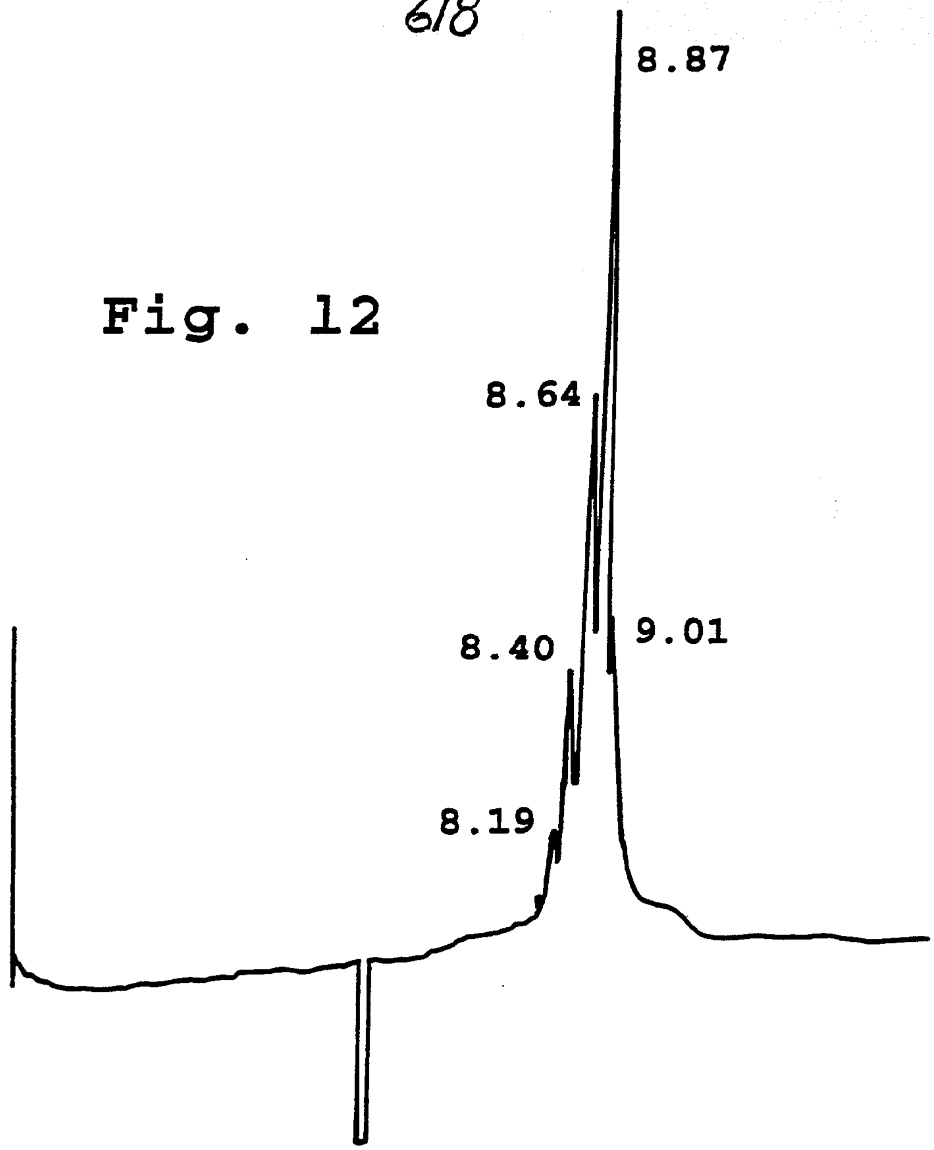
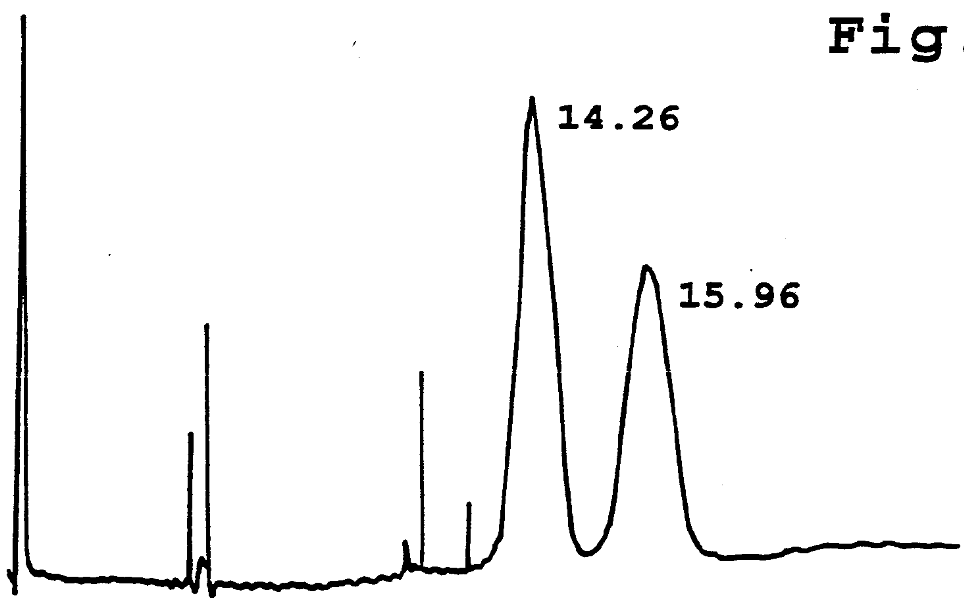


Fig. 13



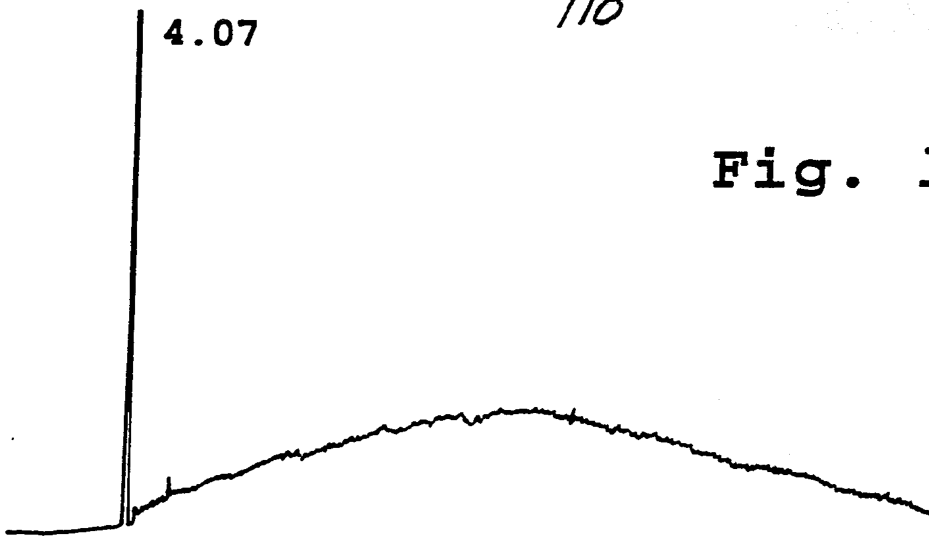


Fig. 14

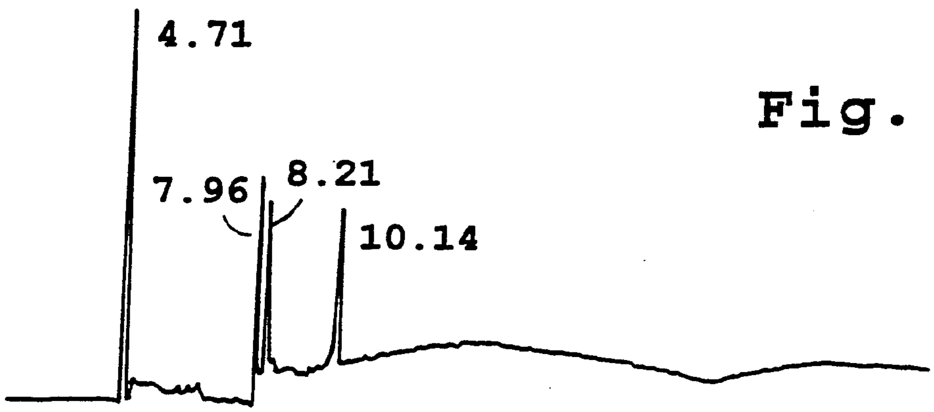


Fig. 15

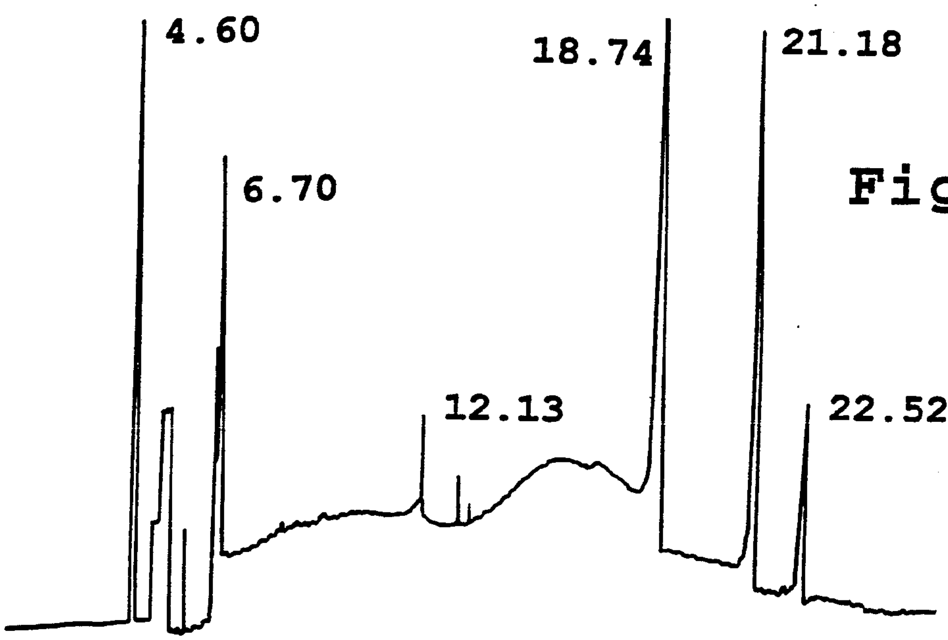
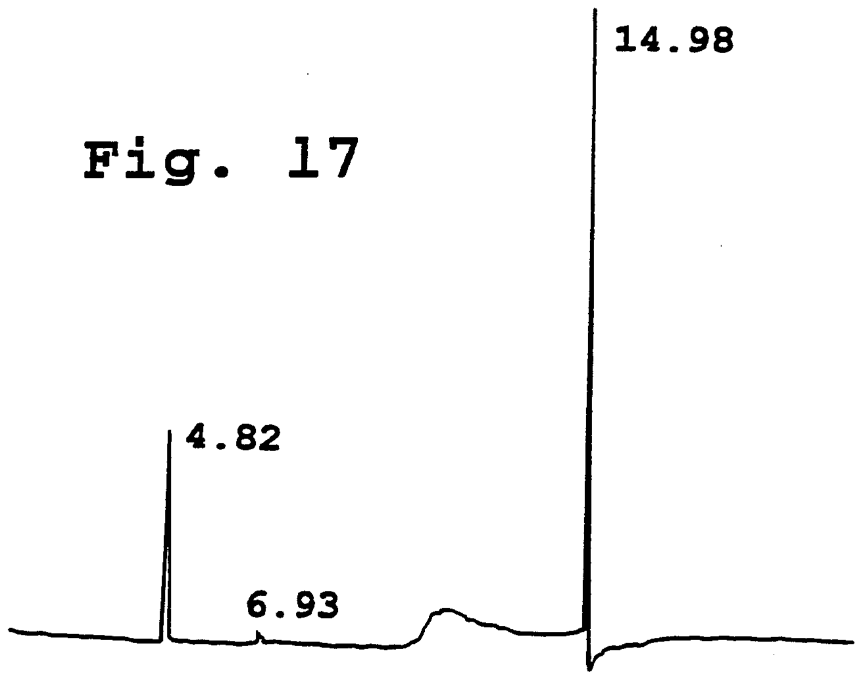


Fig. 16

Fig. 17



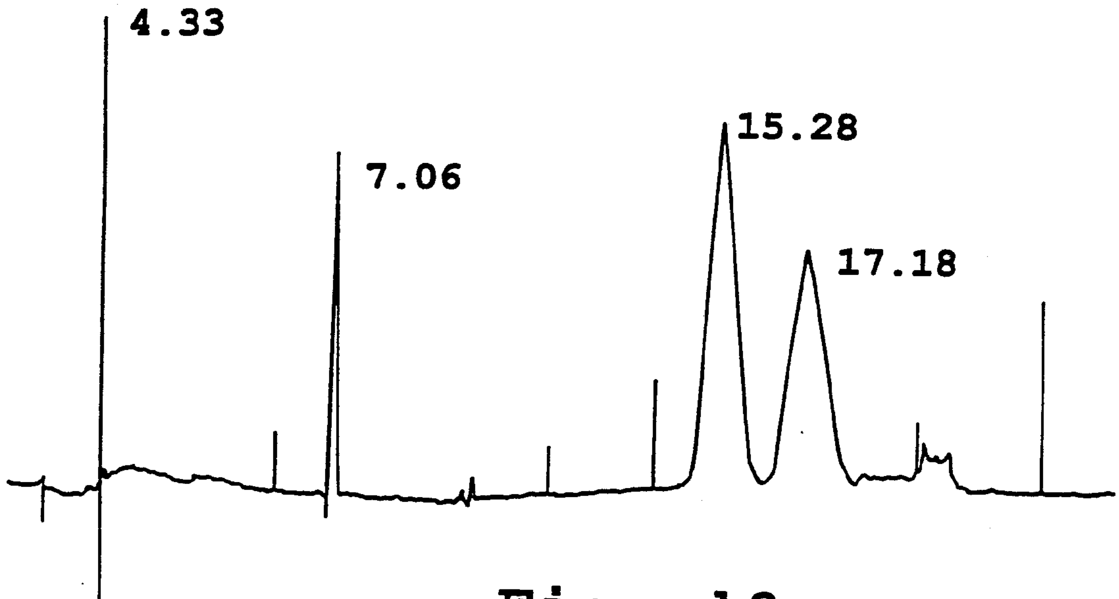


Fig. 18

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/06435

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): G01N 27/26

U.S. CL: 204/180.1,180.7,181.4,182.2,182.8,183.3,299R; 427/230

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	204/180.1,180.7,181.4,182.2,182.8,183.3,299R; 427/230

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
A	US, A, 3,909,380 (DAY) 30 September 1975 (See entire document).	1-20
A X	US, A, 4,680,201 (HJERTEN) 14 July 1987 (See entire document).	1-16 17-20
A X	US, A, 4,865,706 (KARGER) 12 September 1989 (See entire document).	1-16 17-20
A X	US, A, 4,865,707 (KARGER) 12 September 1989 (See entire document).	1-16 17-20

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
04 JANUARY 1991	22 FEB 1991
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	Ngoc-Ho Nguyen Fu NGUYEN NGOC-HO DAVID G. RYSER INTERNATIONAL DIVISION

Form PCT/ISA/210 (second sheet) (May 1986)